TEMPERATURE RELATIONSHIPS OF POIKILOTHERMS AND THE MELTING TEMPERATURE OF MOLECULAR COLLAGEN

B. J. RIGBY

CSIRO Wool Research Laboratories, Division of Textile Physics, Ryde, Sydney, Australia

Biochemical and biophysical data are beginning to accumulate for various proteins of a wide range of animals. These data include the amino acid analyses, molecular weights and dimensions of the molecular units, as well as their thermal and chemical properties. Of these, the protein of connective tissue, collagen, is probably the most widely studied from a comparative point of view (Harkness, 1961; Gross, 1963). It is the main fibrous component of the skins and tendons of the vertebrates and occurs in animals of most of the other phyla. It can be prepared in a fairly pure form from the tissues of most animals and is identified by its characteristic high angle x-ray diagram and its distinctive amino acid composition. In the native state (*e.g.*, tendon, skin, cuticle) or in its molecular state [1] in dilute solution, it exhibits fairly sharp, reproducible melting points. The melting of collagen is predominantly a first order phase transition (Garrett and Flory, 1956), and as such is virtually independent of time and takes place over a small temperature range.

These melting points, *i.e.*, the bulk melting temperature T_S and the molecular melting temperature T_D, are known to correlate with the upper limit of the environmental temperature (where these are available) of the animal concerned (Gustavson, 1956; Leach, 1957; Rigby, 1967a). Thus, all mammals with body temperatures similar to man have the same molecular melting temperature in water, $\backsim 36°$ C. (body temperature 37° C.) while the collagen of cod (a cold-water fish) melts at 15° and that of tuna fish (warm water) melts at 27° C.

For many collagens, T_S and T_D also correlate with the sum of two of the amino acid residues present in the molecule—proline and hydroxyproline (see Harrington and von Hippel, 1961). However it has recently been found (Rigby, 1967a; Fujimoto and Adams, 1964) that some earthworms and the parasitic worm *Ascaris* each contain two distinct collagens with respect to amino acid composition, particularly the sum of proline and hydroxyproline, yet T_S and T_D for each pair is identical (Rigby, 1967a).

It is perhaps advisable at this stage to describe one of these collagens as "collagenous" since it differs in some respects from the generally accepted definition of a collagen. Thus the cuticle of these worms (the collagen in question) while it shows the typical high angle x-ray diagram, and has an amino acid

[1] The molecular unit in vertebrate collagen is composed of three polypeptide chains, each in the form of a left-handed helix wound into a super helix about a common axis. The dimensions of this unit are *ca.* 2800 Å × 14Å, and the molecular weight is *ca.* 300,000 (see Harrington and von Hippel, 1961). The dimensions and molecular weights of invertebrate collagens appear to be different and not yet fully characterized (Maser and Rice, 1962; Josse and Harrington, 1964).

composition similar in pattern to vertebrate collagen, is secreted by epidermal cells rather than by fibroblasts, and does not exhibit the 600–700 Å axial repeat in the electron microscope. However from the point of view of this paper it is not crucial whether the cuticular protein is a true collagen or not, since, as we have already mentioned, it has the same values for T_S and T_D as the body collagen of the same worm (which collagen has all the accepted characteristics of collagen).

In this paper we should like to point out correlations between the melting properties of the collagens, and the behavior and physiological properties of a number of animals, particularly earthworms and parasitic worms. It is not meant to suggest that there is any causal relation between the melting of collagen and sudden changes in physiological behavior (although this is possible), but to emphasize the fact that the thermal properties of at least two proteins allow one to predict some of the temperature relations of the animal.

Materials and Results

Bulk collagen was prepared from the following worms: *Ascaris lumbricoides* and *Macracanthorynchus hirudinaceus* which are parasitic worms from the small intestine of the hog, and three earthworms, *Allolobophora caliginosa, Digaster longmani* and *Pheretima megascolidioides. Digaster* is a giant earthworm found in the Kyogle district of New South Wales and attains a length of 5 feet or more. *Pheretima* is a common Japanese worm and was kindly supplied by Dr. K. Inukai, of the Department of Chemistry, Kyoto University, Japan.

All these worms with the exception of *Macracanthorynchus* have an outer covering, the cuticle, which is almost pure collagen (with the reservation as to definition noted above). This was used as one sample. After removal of the cuticle, collagen can be prepared from the body wall of the worms. This was the second source of collagen from each worm. In the case of *Macracanthorynchus* the body wall could easily be separated into two layers after soaking strips of wall in water overnight. The inner layer is collagen (high angle x-ray, 600–700 Å repeat and amino acid composition) (Rigby, 1967a). The method of purification of each tissue consisted in successive extraction for 24 hours in 0.1% trypsin, 10% NaCl and saturated NaH_2PO_4 solution, then washing in 0.9% saline. Full details of the preparative procedures, purification, identification and chemical composition of these samples have been given elsewhere (Rigby, 1967a).

The thermal stability of each collagen was determined by measuring the melting point of the bulk material in physiological saline, and the melting point of the bulk material in hydrochloric acid at pH 1. The melting point in saline, denoted by T_S, is commonly referred to as the shrinkage temperature. The melting point in HCl at pH 1 has been shown (Rigby, 1961, 1967b) to agree with the temperature of the helix coil transition of molecular collagen in dilute aqueous solution at neutral pH. This temperature is denoted by T_D. The principle of our method is the detection of the sudden increase in force when it melts, in a sample which is prevented from changing its dimensions. In saline it is crystalline aggregates of molecular units which melt, while in acid solution, swelling of the structure and subsequent dispersion of molecular units allows single molecules or small groups to melt.

The results relevant to this paper are given in Table I. The temperatures are the means of at least four determinations; the error is ±1° C.

At this point it is worth noting that for a number of soft collagenous tissues T_S and T_D are independent of the degree of purification. Thus, in the present case, strips of fresh earthworm body wall (*i.e.*, with cuticle removed) give the same values for T_S and T_D as does the collagen extracted from same by the methods described above. While it does not follow that only the collagen is melting it indicates that in soft tissue, at least, some of the physical properties of collagen are unaffected by its association with other components. This point is of particular physiological interest as the following discussion attempts to show.

The question as to the correlation of T_S and T_D with amino acid composition—in particular, with the total pyrrolidine content—is not considered to be relevant to this discussion, but it has been dealt with fully elsewhere (Rigby, 1967a).

TABLE I

Melting temperatures in physiological saline (°C.) of the collagen of various worms. T_S is the melting point of bulk, native sample, T_D the melting point of molecular unit. B, body. C, cuticle. These transitions were measured on the bulk native material (Rigby, 1961, 1967b)

	P. megascolidioides	*Digaster longmani*		*A. caliginosa*		*As. lumbricoides*		*Macracanthorynchus*
	C	C	B	C	B	C	B	B
T_S	37	34	40	34	40	56	59	62
T_D	22	22	22	22	22	40	38	42

DISCUSSION

The first thing to be noticed from Table I is that all the earthworms have the same value of T_D for both cuticle and body collagen. Also, the hog parasitic worms and hog intestinal wall collagens all have similar values of T_D. The values of T_S tend to be the same for each group as well, although a number of factors affect T_S without affecting T_D. In any case it is of more fundamental interest to consider T_D. It will be seen that T_D for the parasitic worms is close to that of their environmental temperature, namely that of the hog. This does not appear to be a coincidence, for it is well known that the T_D values of the collagens of the mammals, man, rat, sheep, and ox, are all virtually the same as that of their deep body temperature, as was mentioned in the introduction. This information is summarized in Figure 1 for a wide range of animals and environments. The simplest explanation of this correlation is of course, natural selection, although why the two temperatures should almost coincide is a question which requires further study. It might be thought, for example, that T_D would be well above the maximum environmental temperature.

The second point of interest in Table I is the value of 22° C. for T_D of all the earthworm collagens. According to Figure 1 this would imply an upper limit of about 22° C. for the environmental temperature of these worms in their natural habitat. There are two kinds of evidence in the literature which are

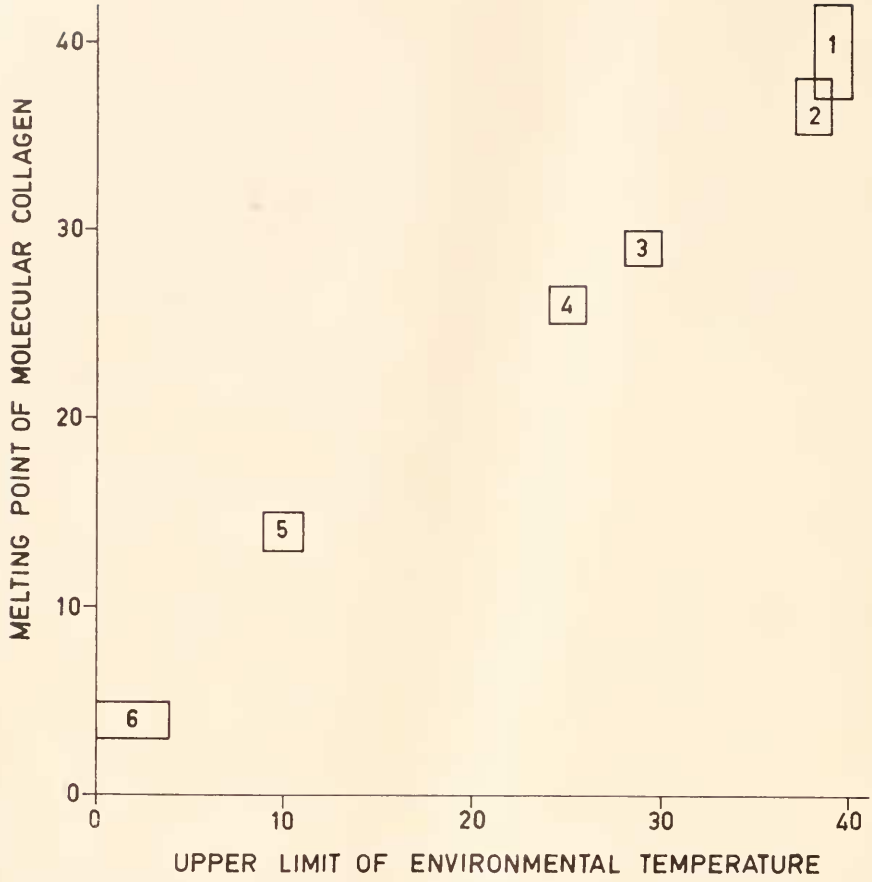

FIGURE 1. The relation between the melting point (T_D), of the collagen molecule from various animals, and the approximate temperature of the upper limit of their environment. 1. *Macracanthorynchus, Ascaris* and hog; 2. rat, human, cow; 3. snail (Newell, 1966) (*Helix aspersa*); 4. tuna skin (Dakin, 1953); 5. cod skin (Tait, 1952); 6. Antarctic "ice-fish." The reference numbers are for environmental temperatures. The Antarctic "ice-fish" is the subject of a detailed paper in preparation.

consistent with this view. These are (i) the thermal preference range of the worm and (ii) the relation between the environmental temperature and the body temperature of the worm. We have only been able to examine collagens of two of the worms studied by others: viz: *Pheretima megascolidioides* and *Allolobophora caliginosa*. In both cases we must assume that temperature was the only factor under observation and secondly, point out that our *Allolobophora* were of Australian origin while the published work to be discussed was performed with North American and Egyptian animals.

We turn first to studies of temperature preference ranges and consider *A. caliginosa* particularly, since we have data for the collagen of this animal. Grant (1955) and El-Duweini and Ghabbour (1965) have studied the thermal

preference ranges of this worm, as well as *E. foetida* and *P. hupeiensis* and *P. californica* and *Alma* sp., respectively. Although the lower limit of this range varies, Grant (1955) quotes 23° C. as the upper limit of each animal. The results of El-Duweini and Ghabbour (1965) are more difficult to interpret since they used *A. caliginosa* from two regions of Egypt. Both groups of worms showed a wide temperature preference range but in both cases there was a sharp drop in the number of worms preferring temperatures above 28° C., this number was about 10% of the total. For *Alma* sp. 88% of the population preferred the range 23–28° C. with the peak at 25° C. *Ph. californica* were almost equally distributed between 26° C. and 35° C. Grant (1955) allowed 24 hours before deciding on a worm's temperature preference, whereas El-Duweini and Ghabbour (1965) allowed 0.5 hour and it could be questioned whether theirs are equilibrium results. However for *A. caliginosa* a T_D value of 22° C. appears to be correlated with the temperature of the upper limit.

Considering next body temperature, we draw attention to the work of Kim (1930) who studied *Pheretima megascolidioides*. His main conclusion was that between 6° and 23° C. the body temperature of *Pheretima* was the same as that of the surrounding medium, but beyond 24° C., for unknown reasons, its body temperature varied erratically. His results imply an upper limit of temperature preference of 23–24° C., and this again (as with *A. caliginosa*) correlates with T_D.

Further, on the subject of sudden physiological changes due to temperature increase such as the one just described, it is relevant to mention the results of Hatai (1922) concerning muscular contraction in several Japanese earthworms. He found rhythmic contraction to be normal and unaffected by temperature up to 23° C., beyond which the form of contraction altered suddenly.

The preceding results and discussion show that earthworms from a number of species and habitats have collagens with identical thermal stability, and, in turn, similar upper limits for their temperature preference range. This value, 22° C., may be characteristic of the family.

It is now of interest to examine the so-called upper lethal temperature. The definition of this temperature is arbitrary and values quoted for a given animal are thus widely spread. Assuming as before that the upper lethal temperature depends only upon temperature and that other factors, such as water content, are kept constant it would seem that the upper lethal temperature could be defined by extrapolation of a time-temperature relation from high temperatures until time and temperature become independent. In other words, above the true lethal temperature, time and temperature would be approximately inversely proportional; below, no relation would be evident.

In the literature on lethal points, El-Duweini and Ghabbour (1965) quote 39° C. for *A. caliginosa* with similar figures for *Ph. californica* and *Alma* sp. Their definition was the temperature at which more than half the sample dies after an exposure of 0.5 hour. Wolf (1938) and Hogben and Kirk (1944), using *L. terrestris* and times of 400 minutes and 12 hours, respectively, gave lethal temperatures of about 29° C. Grant (1955) using an interpolation method and exposure times up to 48 hours gives values of 26° C., 25° C. and 23° C. for *A. caliginosa*, *P. hupeiensis* and *E. foetida*, respectively.

It would appear that the high values quoted by El-Duweini and Ghabbour

(1965) are due to the short exposure time and the inverse relation between time and temperature discussed above, although a factor could be that the worms have some measure of acclimatization to the hot Egyptian climate as mentioned by these authors. Of greater significance, however, is the fact shown by Table I that the thermal shrinkage of earthworm bulk collagen takes place between 35 and 40° C. The values of Grant (1955) are quite close to the upper limit of the preferred temperature range and this is consistent with the data of Kim (1930) where it was seen that above this upper limit definite physiological irregularities become apparent. In other words irreversible changes have begun to occur in the animals' tissues, but long times may still be required before death occurs. One further point which should be mentioned here is that whereas any molecular melting which takes place in bulk collagen is reversible as long as there is no mechanical stress present and T_D is not exceeded by 5–10° C., the melting which occurs at T_S is irreversible except, possibly, in the case of cross-linked collagens such as elastoidin. Thus, so far as collagen is concerned the temperature could exceed T_D for short periods of time without necessarily affecting the animal adversely.

Summarizing, we can say that for the earthworms discussed, the melting point of the collagen molecule (the basic unit of their connective tissue) is very close to the value of the upper limit of their preferred temperature range and in turn to the upper lethal temperature. They are similar to mammals in this respect and to parasitic worms of mammals which at some stage of their life cycle exist at much lower temperatures. Whether or not collagen is uniquely involved in determining these limits cannot be said at this stage, although the fact that it is a protein with a very low rate of turnover (with certain exceptions, Harkness, 1961) and sharp melting point is perhaps significant. It should be pointed out, however, that it is likely that all the animal proteins begin to denature at temperatures above T_D. Finally it has recently been shown by Newell (1966) that the oxygen uptake of various poikilotherms increases abruptly at temperatures which correspond with the upper limit of the environmental temperature, and in one case for which collagen has been isolated (*Helix aspersa*), to the T_D for this collagen, *viz.* 27° C. (Rigby 1967b).

Summary

The molecular unit of collagen, the main fibrous protein of connective tissue, undergoes a first order phase transition at a characteristic temperature, when heated in physiological saline. For the collagen of mammals this temperature is known to almost coincide with the deep body temperature of the animal, and for a number of poikilotherms, with the upper limit of their environmental temperature. We have measured the transition temperature of the collagens of a number of worms and now report that:

1. The molecular unit of the collagen of two hog intestinal worms undergoes this transition at temperatures close to that of the body of their host.

2. For a number of earthworms for which there are precise data on thermal preference limits, the molecular unit of the collagen of these animals has a transition temperature which is very close to the upper limit of the thermal preference zone.

3. There is no evidence of any causal relation between the transition temperature of molecular collagen, and the abrupt physiological and behavioral changes which become apparent at the same temperature. However, the results are direct evidence of a specific protein having easily measurable thermal properties which reflect thermal properties of the whole animal.

LITERATURE CITED

Dakin, W. J., 1953. Australian Seashores. Angus and Robertson, Sydney.

El-Duweini, A. K., and S. L. Ghabbour, 1965. Temperature relations of three Egyptian oligochaete species. *Oikos*, **16**: 9–15.

Fujimoto, D., and E. Adams, 1964. Intraspecies composition difference in collagen from cuticle and body of *Ascaris* and *Lumbricus*. *Biochem. Biophys. Res. Commun.*, **17**: 437–442.

Garrett, R. R., and P. J. Flory, 1956. Evidence for a reversible first-order phase transition in collagen-diluent mixtures. *Nature*, **177**: 176–178.

Grant, W. C., 1955. Temperature relationships in the megascolecid earthworm *Pheretima hupeiensis*. *Ecology*, **36**: 412–417.

Gross, J., 1963. Comparative Biochemistry of Collagen. *In*: Comparative Biochemistry. M. Florkin and H. S. Mason, Eds., Academic Press, N. Y., Vol., 5 pp. 307–346.

Gustavson, K. H., 1956. The Chemistry and Reactivity of Collagen. Academic Press, N. Y.

Harkness, R. D., 1961. Biological functions of collagen. *Biol. Rev.*, **36**: 399–463.

Harrington, W. J., and P. H. von Hippel, 1961. The structure of collagen and gelatin. *Adv. Prot. Chem.*, **16**: 1–138.

Hatai, H., 1922. Contribution to the physiology of earthworms. I. The effect of heat on rhythmic contractions in several species of Oligochaeta. *Jap. J. Zool.*, **1**: 10.

Hogben, L., and R. L. Kirk, 1944. Studies on temperature regulation. I. The Pulmonata and Oligochaeta. *Proc. Roy. Soc.* B, **132**: 239–252.

Josse, J., and W. F. Harrington, 1964. Role of pyrrolidine residues in the structure and stabilization of collagen. *J. Mol. Biol.*, **9**: 269–287.

Kim, H., 1930. On the body temperature of the earthworm. *Tohoku Univ. Sci. Rep. Biology*, **5**: 439–448.

Leach, A. A., 1957. The amino acid composition of amphibian reptile and avian gelatins. *Biochem., J.*, **67**: 83–87.

Maser, M. D., and R. V. Rice, 1962. Biophysical and biochemical properties of earthworm-cuticle collagen. *Biochem. Biophys. Acta*, **63**: 255–265.

Newell, R. C., 1966. Effect of temperature on the metabolism of poikilotherms. *Nature*, **212**: 426–428.

Rigby, B. J., 1961. Two-stage contraction in rat-tail tendon. *Biochem. Biophys. Acta*, **47**: 534–538.

Rigby, B. J., 1967a. Relation between the shrinkage of native collagen in acid solution and the melting temperature of the tropocollagen molecule. *Biochem. Biophys. Acta*, **133**: 272–277.

Rigby, B. J., 1967b. Thermal transitions in some invertebrate collagens and their relation to amino acid content and environmental temperature. *In:* Symposium of Fibrous Proteins, Canberra (Australia). Butterworth and Co. (Aust.).

Tait, J. B., 1952. Hydrography in Relation to Fisheries. Edward Arnold & Co. London.

Wolf, A. V., 1938. Notes on the effect of heat in *Lumbricus terrestris*. *Ecology*, **19**: 346–348.